Original Article
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Obesity

Reduced Expression of Chemerin in Visceral Adipose Tissue Associates with Hepatic Steatosis in Patients with Obesity

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Objective: This study aimed to evaluate whether circulating levels and/or visceral adipose tissue (VAT) expression of recently described adipokines associate with histopathological severity of nonalcoholic fatty liver disease (NAFLD), independent of obesity and insulin resistance.
Methods: Serum levels of adiponectin, omentin, chemerin, monocyte chemoattractant protein-1, and secreted frizzled-related protein 4 were measured using enzyme-linked immunosorbent assay in 81 patients with obesity and NAFLD and 18 lean control subjects. Expression in VAT was measured using real-time PCR and histopathological grading was scored using the NAFLD activity score (NAS).
Results: When NAFLD patients were subdivided into groups with simple steatosis, borderline nonalcoholic steatohepatitis (NASH), and NASH, adiponectin serum levels and omentin expression were lower in NASH versus simple steatosis patients. Serum adiponectin was generally lower with higher histopathological grading. Chemerin VAT expression was negatively associated with NAS ($r = -0.331$, $P = 0.022$) and steatosis score ($r = -0.335$, $P = 0.020$), independent of age, BMI, and HOMA-IR. In addition, adjusting for chemerin VAT expression in a multivariate model explained part of the association between NAS and HOMA-IR.
Conclusions: These findings suggest that lower VAT expression of chemerin in patients with obesity may be involved in the pathophysiology of hepatic steatosis, potentially by modulating the link between insulin resistance and NAFLD.

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Introduction

The growing epidemic of obesity has led to a simultaneously increased prevalence of nonalcoholic fatty liver disease (NAFLD), often referred to as the hepatic manifestation of metabolic comorbidity (1). NAFLD is a chronic liver disease that encompasses a broad histopathological spectrum ranging from simple steatosis (SS) to nonalcoholic steatohepatitis (NASH) and is associated with a higher risk of developing cirrhosis and hepatocellular carcinoma (2). To better address this growing health problem, more insight in the pathophysiology is needed.

Recent data have indicated that adipose tissue-derived secretory factors (adipokines) could play a role in the development of NAFLD and influence disease progression (3). Many adipokines have already been described, and several studies have suggested that they play a role in whole-body energy homeostasis and/or inflammatory responses (4), thus possibly influencing metabolic abnormalities in peripheral tissues in persons with obesity. Elevated chemerin levels have been described frequently in patients with metabolic syndrome and have been associated with body mass index (BMI), serum glucose, triglycerides (TG), high-density lipoprotein (HDL) cholesterol levels, and blood pressure (5). Chemerin is also known to be involved in inflammation by inducing chemotaxis and to induce insulin resistance in skeletal muscle cells (6,7). In contrast, adiponectin and omentin have been found to display anti-inflammatory, insulin-sensitizing, and cardioprotective properties (8,9). Monocyte chemoattractant protein-1 (MCP-1), an adipokine of which higher levels are described in obesity and insulin resistance, has

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also been suggested to play a role in hepatic inflammatory and fibrogenic processes (10,11). Finally, secreted frizzled-related protein 4 (SFRP4), a modulator of the Wnt signaling pathway, has also been described as an adipokine (12), and several Wnt pathway components are associated with lipid and glucose metabolism as well as inflammation (13). Overall, hepatic insulin resistance, inflammation, and modulation of the activity of the Wnt signaling pathway have been reported repeatedly in NAFLD patients, suggesting a common link that potentially contributes to obesity-related NASH (1,14,15). In this study, we assessed serum levels as well as visceral adipose tissue (VAT) expression of chemerin, adiponectin, omentin, MCP-1, and SFRP4 in patients with biopsy-proven NAFLD and lean controls. We hypothesized that a disturbed signature of these adipokines may contribute to the progression of SS to an inflammatory and insulin-resistant NASH status. Therefore, we aimed to evaluate whether these adipokines associate with histopathological disease severity, independent of known insulin resistance in obesity.

Methods

Study design and subjects

Ninety Caucasian patients with obesity (57 men and 33 women; mean BMI 41 kg/m^2), aged between 20 and 68, were recruited from the NASH and Hepobster cohort for evaluation in this study. All patients were scheduled for gastric banding or gastric bypass surgery and met the criteria for bariatric surgery of the International Federation for the Surgery of Obesity: BMI $\geq$40 kg/m^2 or BMI $\geq$35 kg/m^2 in conjunction with obesity-induced comorbid conditions. Twenty men with obesity had type 2 diabetes. Liver and VAT biopsies were obtained during surgery and patients with other causes of liver disease, e.g., hepatitis B or C, viral or autoimmune hepatitis, Wilson disease or any drug-induced liver disease, or evidence of excessive alcohol consumption ($\geq$20 g per day), were excluded. Additional exclusion criteria were primary hypogonadism, abnormal thyroid function, malignancies or carcinoma, serum total cholesterol $>$300 mg/dL, and/or TG $>$450 mg/dL. None of the subjects used steroids, insulin, or thiazolidinediones, and oral glucose-lowering medication was discontinued before surgery. For comparison, 18 Caucasian control men (mean BMI 24 kg/m^2; mean age 44 $\pm$ 12 years) from the Hepobster cohort were recruited. These men underwent elective abdominal surgery for adhesiolysis, hernia diaphragmatica, intestinal resection, or Nissen fundoplication, and VAT biopsies were obtained during surgery. Similar exclusion criteria as mentioned above were applied. In addition, all control subjects had overall good health without medication and with normal liver function tests (chronic elevation of transaminase levels indicates liver disease, i.e., $>$1.5 times the upper normal value for $\geq$3 months). The study protocol was approved by the Ethics Committee of Ghent University Hospital and conducted according to the principles of the Declaration of Helsinki. All participants gave their written informed consent.

Anthropometry and biochemical assays

Anthropometric measurements were performed during a preoperative examination. Body weight was measured to an accuracy of 0.1 kg in light indoor clothing without shoes, whereas height was measured using a wall-mounted stadiometer. Blood samples were collected after overnight fasting, before surgery, and were centrifuged, fractionated, and stored at $-$80°C until analysis. Triglyceride and glucose levels were determined colorimetrically (Roche Diagnostics, Mannheim, Germany), and insulin levels were measured using electrochemoluminescent immunoassay (modular immunoassay, Roche Diagnostics). Homeostasis model of the assessment for insulin resistance (HOMA-IR) was calculated with the following formula: (fasting glucose [mmol/L] $\times$ fasting insulin [μU/mL])/22.5. Aspartate aminotransferase (AST), alanine aminotransferase (ALT), γ-glutamyltransferase (GGT), and C-reactive protein (CRP) were routinely measured using a conventional automated analyser.

Measurement of adipokine levels

Serum levels of adipokines were measured using commercial enzyme-linked immunosorbent assay (ELISA) kits, according to manufacturer's instructions. Chemerin, MCP-1, and adiponectin were determined with kits provided by R&D Systems (Minneapolis, MN). The respective intra- and inter-assay coefficients of variation (CV) were 2.8% and 6.4% for chemerin, 4.9% and 4.6% for MCP-1, and 4.7% and 4.9% for adiponectin. Omentin serum levels were assessed using the Human Omentin-1 ELISA kit from BioVendor (Brno, Czech Republic). Intra- and interassay CV were 3.7% and 4.6%, respectively, for omentin. Circulating levels of SFRP4 were measured using the USCN Life Science ELISA kit (Hoelzel Diagnostika, Cologne, Germany). Intra- and inter-assay CV for SFRP4 were $<$10% and $<$12%, respectively.

For gene expression analysis, VAT biopsies were collected from the gastrosplenic or gastrocolic ligaments at the end of surgery from 63 male subjects of the Hepobster cohort (13 controls and 50 subjects with obesity), immediately frozen, and stored at $-$80°C until further use. RNA was isolated from 100 mg of the frozen fat samples with the TriPure Isolation Reagent kit (Roche Diagnostics) according to manufacturer's instructions. Contaminating genomic DNA was removed with RNase-free DNase incubation (Qiagen, Hilden, Germany), followed by a cleanup step with the RNeasy Mini Kit (Qiagen). cDNA synthesis was carried out using the GoScript Reverse Transcription System (Promega, Mannheim, Germany) according to manufacturer's instructions. Expression of MCP-1 and chemerin was quantified by real-time PCR using the following QuantiTect Primer Assays: Hs_CCL2_1_SG (MCP-1, *CCL2* gene; Cat#QT00212730), Hs_RARRES2_1_SG (chemerin, *RARRES2* gene; Cat#QT00091945) (Qiagen). Primers to measure omentin (*INTL1* gene) and SFRP4 expression were TCAGCTTCCTGCTGTTTCTCATA and GGAGAC GAAGAACAGGTCCATT for omentin and CACCCATCCCTCGA ACTCAA and TGTGTGGACACTGGCAAGAAG for SFRP4 (Eurogentec, Köln, Germany). Real-time PCR analysis was carried out on a StepOnePlus system (Applied Biosystems) with GoTaq qPCR Master Mix (Promega). Gene expression levels were calculated from the obtained threshold cycle (Ct) values after normalization for the expression of stable reference genes, *UBE2D2*, *YWHAZ* (Eurogentec), and *RPS18* (QuantiTect Primer Assay, Cat#QT02323251, Qiagen), using qBase Plus software (version 2.6; Biogazelle, Ghent, Belgium).

Hepatic histopathological analysis

Liver biopsies were obtained from all subjects with obesity at the end of surgery, each measuring 5 $\times$ 5 mm, and were taken from the lateral edge of the left liver lobe (segment 3) using bipolar forceps. These were immediately fixated in formalin (buffered 4% paraformaldehyde solution; Klinipath, Belgium) at room temperature for microscopic analysis. Formalin fixed liver biopsies were routinely processed and stained with hematoxylin-eosin and Masson trichrome. An experienced pathologist (MP) established the histological diagnosis of NAFLD according to the scoring system of Kleiner et al. (16), blinded to characteristics of participants. At least six complete portal tracts in

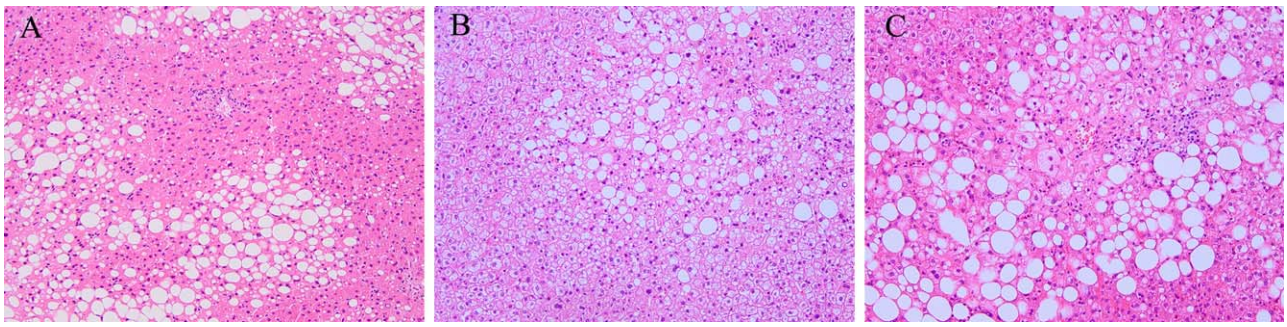

Figure 1 Three different examples of histopathological scoring according to nonalcoholic fatty liver disease (NAFLD) activity score (NAS). (**A**) Example of a low NAS (steatosis, grade 2; ballooning, grade 0; lobular inflammation, grade 0; NAS 2) diagnosed as simple steatosis. (**B**) Example of an intermediate NAS (steatosis, grade 2; ballooning, grade 1; lobular inflammation, grade 1; NAS 4) diagnosed as mild steatohepatitis or borderline nonalcoholic steatohepatitis (NASH). (**C**) Example of a high NAS (steatosis, grade 2; ballooning, grade 2; lobular inflammation, grade 1; NAS 5) diagnosed as definite steatohepatitis or NASH (10×, hematoxylin and eosin). [Color figure can be viewed at wileyonlinelibrary.com.]

liver specimen were required for adequate histological evaluation. Steatosis was judged as the percentage of hepatocytes containing fat droplets, with a minimum of 5%. NAFLD activity score (NAS) is defined as the unweighted sum of scores for steatosis intensity (0–3; <5%, 5–33%, 33–66%, or >66%), lobular inflammation (0–3; no, <2, 2–4, or >4 inflammatory foci), and hepatocellular ballooning (0–2; no, few, or many ballooning cells), ranging from 0 to 8 (Figure 1). Scores of 0 to 2 were considered as SS and scores of >5 were considered as NASH. Intermediate scores of 3 or 4 were considered as borderline NASH (16). Stage of fibrosis was scored using a 4-point scale.

Statistics

Statistical analysis was performed using IBM SPSS Statistics (version 21.0). Data distribution of continuous variables was evaluated with the Kolmogorov-Smirnov test. Normally distributed variables were expressed as mean ± SD, whereas non-Gaussian distributed variables were described as median (interquartile range). Adipokines were compared among the different subject groups, i.e., lean subjects, subjects with obesity but without NAFLD, and subjects with obesity and NAFLD. Furthermore, among NAFLD patients, adipokines were compared between patients with low, moderate, or high levels of histopathological severity, i.e., degree of steatosis, hepatocyte ballooning, lobular inflammation, and fibrosis. For these comparison analyses among the different study groups and histological parameters, ANOVA and Tukey HSD *post hoc* test or Kruskal-Wallis and Mann-Whitney *U* test were applied. Categorical variables were analyzed with the χ^2 test and Fisher's test. Multivariate analysis was performed to identify independent factors associated with disease severity in NAFLD patients. Our findings were adjusted for potential confounders such as age, BMI, and insulin resistance, using multivariate linear regression analysis (ENTER model).

Results

General characteristics, adipokine serum levels, and VAT expression in controls and NAFLD patients

A total of 108 subjects were included in the study cohort (75 men and 33 women), including 18 normal-weight controls, 9 patients with obesity but without NAFLD, and 81 patients with obesity and biopsy-proven NAFLD. NAFLD patients had higher BMI, insulin levels, HOMA-IR, ALT, GGT, and CRP levels compared with normal-weight controls, while there were no differences in TG and AST levels. Twenty-five percent of NAFLD patients had type 2 diabetes, whereas none of the controls or patients with obesity but without NAFLD had diabetes (Table 1).

Serum adiponectin levels were lowest in NAFLD patients compared with normal-weight controls and obesity patients without NAFLD ($P = 0.004$ and 0.020, respectively), whereas serum MCP-1 levels were lowest in the obesity without NAFLD group versus controls and NAFLD patients ($P < 0.05$). Chemerin serum levels were higher in NAFLD patients compared with normal-weight controls ($P = 0.020$). Circulating omentin and SFRP4 levels were not different among the groups. With regard to adipokine VAT expression, omentin and SFRP4 were higher ($P = 0.043$ and <0.001, respectively), and chemerin tended to be lower in NAFLD patients compared with normal-weight controls ($P = 0.053$). MCP-1 expression was similar between control and NAFLD patients (Table 1).

General characteristics and adipokine levels according to disease severity in NAFLD patients

Potential differences in circulating adipokine levels and VAT expression were examined in relation to disease severity. From the 81 patients with obesity diagnosed with NAFLD, 32 subjects had SS, 24 subjects borderline NASH, and 25 subjects confirmed NASH. General characteristics as well as adipokine levels among groups are listed in Table 2. There were no differences in gender, age, BMI, fasting glucose and insulin, TG, ALT, and CRP levels among the groups. HOMA-IR, AST, and GGT levels were higher in both borderline and confirmed NASH patients as compared with those with SS ($P = 0.033$, 0.023, and 0.002, respectively). There were no clinical or biochemical differences between borderline NASH and NASH patients.

Serum levels of adiponectin were lower in NASH patients compared with patients with SS ($P = 0.030$). The other serum adipokines were not different among the three NAFLD groups. Similarly, there were no differences in VAT expression of adipokines among the

TABLE 1 General characteristics of healthy controls, patients with obesity without NAFLD, and patients with obesity and NAFLD

Parameter	Healthy controls ($N = 18$)	Obesity patients without NAFLD ($N = 9$)	NAFLD patients ($N = 81$)	P
General characteristics				
M/F	18/0	2/7$^{***\#\#}$	55/26**	<0.001
T2D	0	0	20 (25%)*	0.017
Age (years)	44 ± 12	45 ± 9	45 ± 10	0.995
BMI (kg/m^2)	24 [22–26]	36 [34–41]$^{***\#}$	41 [38–44]***	<0.001
Glucose (mmol/L)	5.44 [4.63–5.84]	5.11 [4.78–5.86]	5.33 [4.78–6.31]	0.766
Insulin (pmol/L)	25.7 [21.1–40.4]	57.1 [35.8–111.1]**	92.9 [57.5–150.9]***	<0.001
Triglycerides (mg/dL)	156.0 [111.5–204.9]	104.0 [70.5–139.5]$^{*\#\#\#}$	185.0 [144.0–241.0]	<0.001
HOMA-IR	0.87 [0.60–1.47]	1.79 [1.04–3.89]**	3.21 [1.92–6.10]***	<0.001
AST (IU/L)	22.0 [17.8–33.0]	20.0 [15.5–22.0]$^{\#}$	28.0 [20.0–40.0]	0.023
ALT (IU/L)	11.3 [8.5–29.5]	21.0 [9.0–26.5]	25.0 [14.5–36.5]*	0.036
GGT (U/l)	22.2 [16.0–29.2]	15.0 [10.5–31.0]$^{\#}$	30.0 [19.5–50.5]*	0.009
CRP (mg/L)	1.05 [0.60–2.20]	0.50 [0.15–5.30]	3.00 [1.10–5.35]*	0.046
Serum adipokine levels				
Omentin (ng/mL)	398.2 [336.0–490.3]	394.9 [263.4–534.8]	391.6 [307.1–480.7]	0.894
Adiponectin (mg/mL)	6.15 [5.30–10.89]	9.45 [4.10–12.54]$^{\#}$	4.28 [2.94–7.11]**	0.002
Chemerin (ng/mL)	164.7 [112.1–211.0]	177.5 [155.2—235.4]	200.3 [146.6–256.1]*	0.063
MCP-1 (pg/mL)	288.6 [239.5–354.0]	210.1 [192.0–234.8]$^{*\#\#}$	299.5 [252.4–395.6]	0.010
SFRP4 (pg/mL)	4569.0 ± 2833.8	4255.6 ± 2351.4	4650.8 ± 1805.0	0.709
Adipokine VAT expression, AU				
N	15		48	
Omentin	1.42 [0.07–13.98]		6.17 [1.98–22.28]*	0.043
Chemerin	1.09 [0.81–1.46]		0.87 [0.62–1.07]	0.053
MCP-1	1.91 [0.20–5.05]		0.58 [0.36–1.59]	0.488
SFRP4	1.01 [0.67–1.23]		2.86 [1.75–4.32]***	<0.001

Data are presented as mean ± SD or median [interquartile range] and were analyzed using Mann-Whitney U test and χ^2 test for categorical variables.
*Versus healthy controls; # versus NAFLD patients.
$^{*,\ \#}P < 0.05$; $^{**,\ \#\#}P < 0.01$; $^{***,\ \#\#\#}P < 0.001$.
ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; CRP, C-reactive protein; GGT, gamma-glutamyltransferase; HOMA-IR, homeostasis model of the assessment for insulin resistance; M/F, male-to-female ratio; MCP-1, monocyte chemoattractant protein-1; NAFLD, nonalcoholic fatty liver disease; SFRP4, secreted frizzled-related protein 4; T2D, type 2 diabetes; VAT, visceral adipose tissue.

groups, except for lower omentin expression in patients with NASH versus patients with SS ($P = 0.043$).

Associations between adipokines and histopathological grading

We next examined whether adipokine levels and VAT expression were related to the various histopathological states. As shown in Figure 2, serum adiponectin levels ($r = -0.308$, $P = 0.005$; Figure 2A), and chemerin VAT-expression ($r = -0.331$, $P = 0.022$; Figure 2B) negatively associated with NAS. The other adipokines were examined similarly and showed no significant relations with NAS.

When considering the individual histopathological parameters, it was found that serum adiponectin levels and chemerin VAT expression were lower in patients with moderate or severe steatosis (>33%) versus patients with mild steatosis (<33%) ($P = 0.001$ and 0.007, respectively; Figures 3 and 4). Furthermore, serum adiponectin was lower in patients with higher grades of hepatocyte ballooning, lobular inflammation, and fibrosis (all $P < 0.05$; Figure 4). There were no differences in levels of other adipokines, both serum levels and VAT expression, according to histopathological grading.

Finally, we investigated whether serum adiponectin and chemerin VAT expression function as independent predictors of NAS in addition to known confounders such as HOMA-IR and BMI. Among these patients with NAFLD, BMI was similar in patients with different grades of histopathological parameters and did not correlate with overall NAS. In contrast, HOMA-IR was higher in patients with higher grades of steatosis, hepatocyte ballooning, and lobular inflammation (all $P < 0.05$) and was positively associated with NAS ($r = 0.285$, $P = 0.010$).

Multivariate linear regression analysis was performed to identify independent predictors of NAS in NAFLD patients (Table 3). Whereas the association between HOMA-IR and NAS was independent of sex, age, and BMI (model 1), it lost significance after correcting for chemerin VAT. Chemerin VAT expression remained negatively associated with NAS, independent of age, BMI, and HOMA-IR in male NAFLD patients (model 2), whereas adiponectin

TABLE 2 Clinical and biochemical characteristics of NAFLD patients with obesity plus simple steatosis, borderline NASH, and NASH

Parameter	SS ($N = 32$)	Borderline NASH ($N = 24$)	NASH ($N = 25$)	P
Histological grading according to NAS				
NAS	1-2	3-4	5-8	<0.001
Steatosis grade (0/1/2/3)	0/26/6/0	0/8/15/1	0/0/5/20	<0.001
Lobular inflammation (0/1/2/3)	27/5/0/0	4/20/0/0	2/16/6/1	<0.001
Hepatocellular ballooning (0/1/2)	31/1/0	2/21/1	0/7/18	<0.001
Fibrosis (0/1/2/3/4)	23/8/1/0/0	6/11/7/0/0	2/12/6/3/1	<0.001
Clinical and biochemical characteristics				
M/F	19/13	17/7	19/6	0.384
T2D	4 (12.5%)	10 (42%)	6 (24%)	0.043
Age (years)	43 ± 11	47 ± 11	46 ± 10	0.360
BMI (kg/m^2)	42 [39–46]	39 [36–44]	42 [37–43]	0.302
Glucose (mmol/L)	5.25 [4.69–5.67]	5.39 [4.74–7.21]	5.56 [4.97–6.58]	0.376
Insulin (pmol/L)	78.0 [47.9–119.9]	107.6 [76.7–158.8]*	103.7 [57.1–229.2]	0.095
Triglycerides (mg/dL)	178.5 [118.8–215.2]	208.5 [161.8–258.5]*	174.0 [143.5–288.5]	0.129
HOMA-IR	2.41 [1.70–4.08]	4.00 [2.20–7.00]*	3.50 [1.97–8.19]*	0.033
AST (IU/L)	24.5 [17.0–33.5]	25.5 [20.5–40.0]	34.0 [25.0–55.7]**	0.023
ALT (IU/L)	21.2 [15.3–34.0]	29.5 [11.5–35.8]	28.0 [10.0–45.4]	0.607
GGT (U/l)	23.2 [16.0–34.8]	32.0 [20.0–54.5]*	41.5 [28.5–66.5]**	0.002
CRP (mg/L)	2.30 [1.10–5.60]	2.10 [0.70–5.18]	4.10 [1.65–5.48]	0.436
Serum adipokine levels				
Omentin (ng/mL)	363.4 [301.8–492.5]	430.3 [313.5–519.7]	353.3 [293.5–437.9]	0.234
Adiponectin (mg/mL)	5.08 [3.56–8.36]	3.99 [2.83–6.39]	4.18 [2.37–4.75]*	0.063
Chemerin (ng/mL)	203.0 [154.0–271.5]	195.9 [147.6–255.7]	202.4 [139.9–232.2]	0.759
MCP-1 (pg/mL)	282.7 [239.9–396.9]	319.9 [274.2–408.5]	300.7 [263.3–381.3]	0.531
SFRP4 (pg/mL)	4725.7 ± 1414.3	4273.4 ± 1754.7	4939.9 ± 2167.4	0.673
Adipokine VAT expression, AU ($N = 48$)				
N	16	16	16	
Omentin	16.78 [3.59–36.15]	5.28 [1.59–26.11]	5.87 [1.63–10.86]*	0.166
Chemerin	0.88 [0.65–1.25]	0.97 [0.66–1.07]	0.69 [0.56–0.92]	0.260
MCP-1	0.52 [0.32–2.18]	0.54 [0.32–1.35]	0.66 [0.40–1.43]	0.896
SFRP4	3.78 [1.85–4.59]	2.54 [1.68–3.96]	2.99 [2.07–4.38]	0.484

Data are presented as number of cases, mean ± SD, and median [interquartile range] and were analyzed using Kruskal-Wallis and Mann-Whitney *U* test.
*versus SS.
*$P < 0.05$; **$P < 0.01$.
ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; CRP, C-reactive protein; GGT, gamma-glutamyltransferase; HOMA-IR, homeostasis model of the assessment for insulin resistance; M/F, male-to-female ratio; MCP-1, monocyte chemoattractant protein-1; NAFLD, nonalcoholic fatty liver disease; NAS, NAFLD activity score; NASH, nonalcoholic steatohepatitis; SFRP4, secreted frizzled-related protein 4; SS, simple steatosis; T2D, type 2 diabetes; VAT, visceral adipose tissue.

serum levels were no longer associated with NAS after correcting for sex, age, BMI, and HOMA-IR (model 3). In a final model, adjusting for adiponectin levels made the relation between chemerin VAT expression and NAS borderline significant ($\beta = -0.284$, $P = 0.072$; model 4).

Discussion

In a group of biopsy-proven NAFLD patients with obesity, we found lower adiponectin and higher chemerin serum levels as compared with healthy control subjects. Adiponectin, but not chemerin serum levels were also associated with disease severity and grade of steatosis. In contrast to the serum levels, chemerin VAT expression appeared inversely associated with NAFLD severity, as expression was significantly lower in patients with higher degrees of steatosis and overall NAS. This finding persisted after adjustment for age, BMI, and HOMA-IR. Further, our results suggest that the well-established link between insulin resistance and NAFLD/NASH might in part be dependent on VAT expression of chemerin.

NAFLD develops when surplus fat accumulates in the liver, which might be both resulting from and contributing to insulin resistance. Indeed, insulin resistance is known to result in an increased hepatic *de novo* lipogenesis, together with an increased delivery of lipids to the liver (17). The prevalence and severity of NAFLD have been

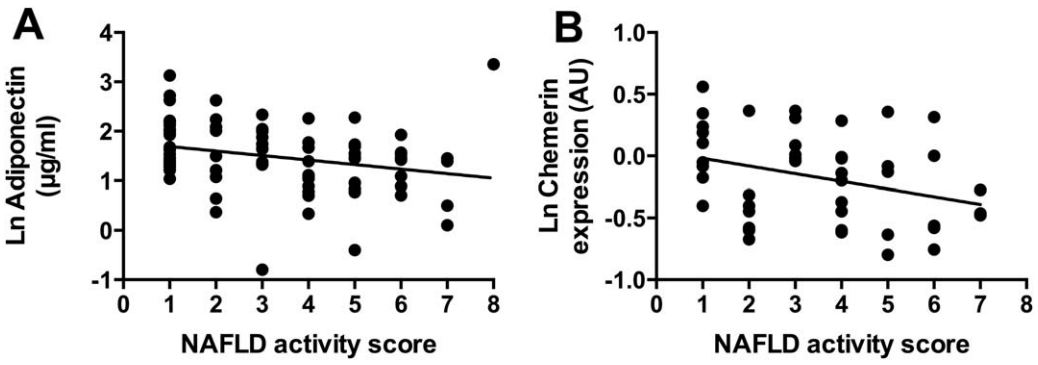

Figure 2 The inverse relationship between disease severity, presented as the nonalcoholic fatty liver disease (NAFLD) activity score (NAS) and (**A**) ln-transformed serum adiponectin ($N = 81$) as well as (**B**) visceral adipose tissue expression of chemerin ($N = 63$) in biopsy-proven NAFLD patients.

strongly related to BMI, waist circumference, hyperinsulinemia, hypertriglyceridemia, impaired glucose tolerance, and type 2 diabetes, which are all metabolic parameters with the common underlying factor insulin resistance (18,19). Although NAFLD severity did not associate with BMI in this study, HOMA-IR was associated with steatosis, hepatocyte ballooning, and lobular inflammation score and with overall NAS, independent of sex, age, and BMI.

Our study also showed an inverse association between serum adiponectin levels and grade of steatosis, lobular inflammation, hepatocyte ballooning, fibrosis, and overall NAS, corroborating previous findings (20). Adiponectin has an antisteatotic effect on hepatocytes by increasing fatty acid oxidation and decreasing gluconeogenesis, fatty acid influx, and *de novo* lipogenesis (21). Additionally, anti-inflammatory and antifibrotic effects of hepatic adiponectin have been reported (22). Recently, a meta-analysis confirmed lower adiponectin levels in NASH/NAFLD compared with controls (23). The few prospective studies with paired liver biopsies, however, reported conflicting results and could not always associate adiponectin with NAFLD progression (24,25). In our multivariate analysis, the association between NAS and adiponectin was lost when HOMA-IR was added to the model, suggesting that insulin resistance might mediate the relation between NAFLD and adiponectin. Since adiponectin is highly associated with insulin resistance, these findings suggest that low circulating levels of adiponectin may contribute to insulin resistance, which in turn may influence NAFLD.

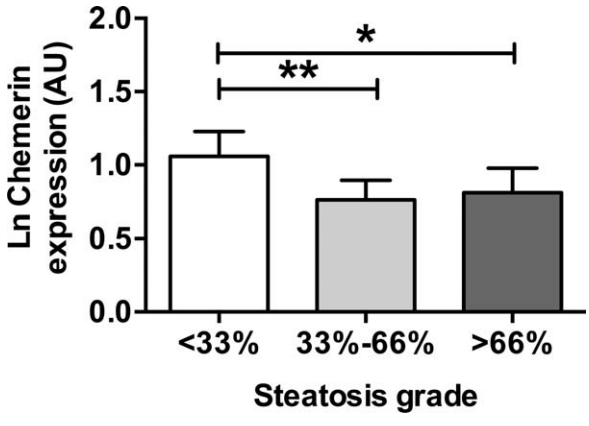

Figure 3 Chemerin VAT expression was inversely associated with steatosis grade ($P = 0.007$; Kruskal-Wallis test). Expression was highest in patients with <33% of steatosis and was lower in patients with moderate (33–66%) to severe (>66%) steatosis grade. $^{*}P < 0.05$; $^{**}P < 0.01$.

Chemerin has been described as a marker of insulin resistance, with both higher serum levels and adipose tissue expression in patients with impaired glucose tolerance or type 2 diabetes (26,27). Chemerin has been reported to interfere with insulin signaling in skeletal muscle cells and to be implicated in the regulation of inflammation, suggesting a potential involvement in NAFLD (6,7). Indeed, several studies have reported elevated serum or hepatic expression levels of chemerin in NAFLD and/or NASH patients and found positive associations with NAS or hepatic inflammation (28,29). In this study, serum chemerin levels were higher in patients with NAFLD and obesity compared with controls; however, they were similar among the three NAFLD groups and were not related to histopathological severity. In contrast, chemerin VAT expression had a tendency to be lower in patients with NAFLD versus controls and was lower in patients with higher grades of steatosis compared with lower steatosis grades. Chemerin expression was moderately negatively associated with NAS, independent of confounding factors. Moreover, the association between HOMA-IR and NAS lost significance after correcting for chemerin VAT expression, suggesting that chemerin may at least partly influence the link between HOMA-IR and NAFLD severity. Recently, Wolfs et al. similarly reported a negative association between chemerin VAT expression and histopathological parameters of NAFLD patients. In their study, chemerin expression was negatively associated with steatosis, lobular, and portal inflammation, independent of obesity, HOMA-IR, and type 2 diabetes (30). Experimental studies have reported inconsistent results, indicating both pro- and anti-inflammatory properties of chemerin. It has been described as a chemoattractant of innate immune cells such as Kupffer cells, which in turn are believed to be key players in the pathophysiology of NAFLD (31). However, while a mouse model with diminished chemerin action showed no effects on insulin resistance and NAFLD compared with wild type (32), the administration of recombinant chemerin attenuated the inflammatory response in another model (33). Although the associations were moderate, the anti-inflammatory effects of chemerin and its negative association with

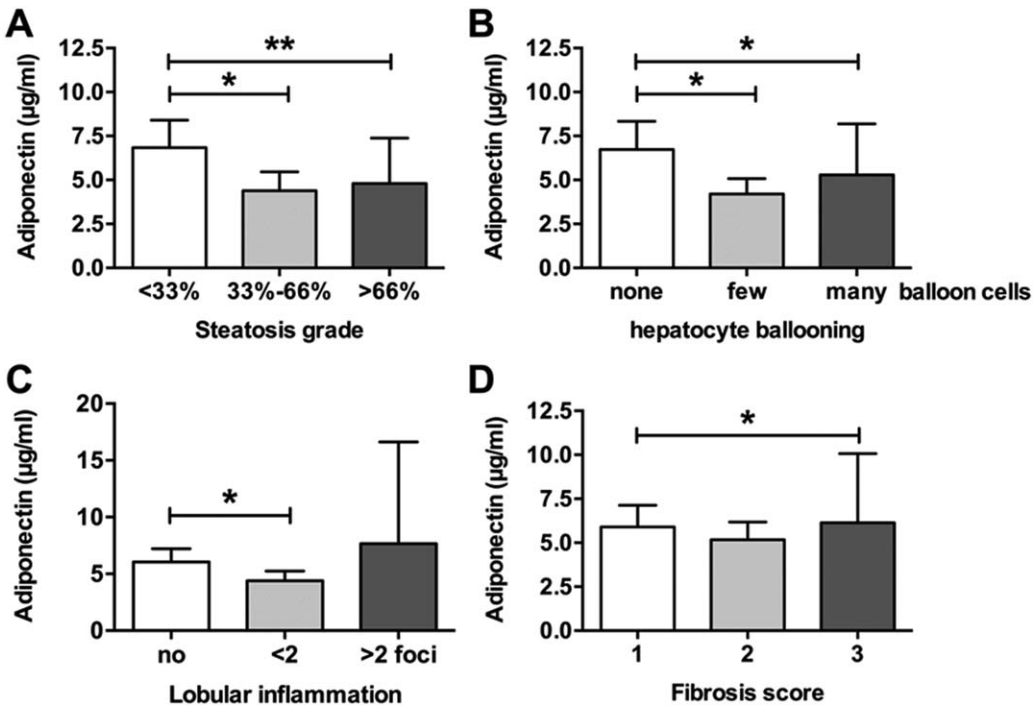

Figure 4 Adiponectin serum levels among the histopathological parameters in NAFLD patients. Serum adiponectin was inversely associated with (**A**) the grade of steatosis, (**B**) hepatocyte ballooning, (**C**) lobular inflammation, and (**D**) fibrosis (P = 0.001, P = 0.033, P = 0.027, and P = 0.042, respectively; Kruskal-Wallis test among groups), with lower levels in patients with higher grades. $^{*}P < 0.05$; $^{**}P < 0.01$.

NAS suggest that this lower VAT expression of chemerin in patients with obesity may be involved in the pathophysiology of NAFLD. Importantly, none of the adipokine serum levels were associated with their respective VAT expression. As the majority of blood flow (±80%) to the liver is delivered via the portal vein, which is closely connected to VAT, it is likely that systemic serum concentrations of adipokines do not accurately reflect hepatic delivery of adipokines. Furthermore, secretion of adipokines is different between subcutaneous adipose tissue (SAT) and VAT, of which SAT is suggested to contribute more to systemic levels. Indeed, Alfadda et al. recently highlighted the differential expression of chemerin between SAT and VAT in subjects with obesity, with higher expression in SAT (34).

TABLE 3 Multivariate linear regression model with NAS as dependent variable in biopsy-proven NAFLD patients

Independent variable	Model 1, $F = 3.028$; $R^2 = 0.139$ β (±SE); P value	Model 2, $F = 3.209$; $R^2 = 0.234$ β (±SE); P value	Model 3, $F = 2.742$; $R^2 = 0.156$ β (±SE); P value	Model 4, $F = 2.678$; $R^2 = 0.246$ β (±SE); P value
Sex	0.244 (±0.476); 0.035	Constant	0.209 (±0.511); 0.090	Constant
Age	0.205 (±0.951); 0.085	0.103 (±1.172); 0.505	0.223 (±0.951); 0.062	0.114 (±1.182); 0.465
HOMA-IR	0.275 (±0.265); **0.020**	0.244 (±0.286); 0.088	0.210 (±0.302); 0.116	0.185 (±0.323); 0.248
BMI	−0.056 (±1.484); 0.643	−0.211 (±2.062); 0.150	−0.047 (±1.477); 0.693	−0.202 (±2.077); 0.170
Chemerin VAT expression		−0.317 (±0.797); **0.038**		−0.284 (±0.830); 0.072
Serum adiponectin levels			−0.135 (±0.379); 0.313	−0.128 (±0.454); 0.422

Independent variables in the different models were: model 1: sex, age, HOMA-IR and BMI; model 2: sex was constant, age, HOMA-IR, BMI, and chemerin VAT expression; model 3: sex, age, HOMA-IR, BMI, and serum adiponectin levels; model 4: sex was constant, age, HOMA-IR, BMI, chemerin VAT expression, and serum adiponectin levels. Data are presented as β coefficients with standard error (SE) of the predicted coefficients (B), and F and R^2 values are given per model. Because visceral adipose tissue (VAT) was only sampled from male patients with NAFLD, sex was constant in model 2 and model 4.
HOMA-IR, homeostasis model of the assessment for insulin resistance; NAS, nonalcoholic fatty liver disease (NAFLD) activity score.

Other adipokines neither associated with histological parameters nor with NAFLD severity in our cohort. VAT expression of omentin and SFRP4 were significantly higher in subjects with obesity versus controls, without differences in serum levels. This counteracts with the description of omentin in literature as an anti-inflammatory and insulin-sensitizing adipokine, of which low serum levels have been reported in patients with obesity, insulin resistance, and type 2 diabetes (35). NAFLD and higher degree of hepatocyte ballooning in biopsy-proven NAFLD patients have previously been positively associated with serum omentin levels (36). Higher levels of SFRP4 have been linked to type 2 diabetes and obesity (37) and could be related to NAFLD because a mouse model with the hepatocyte-specific knockout of the canonical Wnt pathway was more sensitive to develop NASH and fibrosis compared with wild type (38). However, we investigated SFRP4 levels in human NAFLD for the first time and could not confirm this. Finally, although serum MCP-1 levels were lower in the small group of patients with obesity but without NAFLD, serum levels nor VAT expression were associated with histopathological severity. This contrasts with experimental studies suggesting involvement of MCP-1 in hepatic inflammation (10,11) and reports of higher MCP-1 levels in NAFLD patients (39).

This study has some limitations. First, the cross-sectional design does not allow to indicate a causative link or to address the mechanisms behind the observed associations. These results are thus merely suggestive and need further investigation in larger-scaled prospective studies. The use of paired biopsies could allow investigating changes of certain adipokines when NAFLD progresses or regresses over time. Next, the relatively small sample size among study groups limits the generalizability of our results and conclusions. Thirdly, patients with obesity, type 2 diabetes, and NAFLD are closely linked because of the common causative factor insulin resistance, which makes it difficult to distinguish metabolic confounding factors. Furthermore, it is known that VAT, and not SAT, is strongly associated with NAFLD severity (40). In this cohort, adipose tissue distribution was not assessed, which might potentially explain the lack of association between BMI and NAFLD. Finally, although liver biopsy is recognized to be the golden standard to diagnose NAFLD, the chance of sampling error remains inevitable.

In summary, we found that in patients with obesity and NAFLD, disease severity and the degree of hepatic steatosis inversely associate with chemerin VAT expression, independent of insulin resistance. These findings suggest a potential role of lowered chemerin VAT expression in the pathophysiology of hepatic steatosis. Moreover, chemerin VAT expression even appears to modulate the relation between NAFLD severity and insulin resistance. More knowledge on the determinants of chemerin VAT expression might help to elucidate the relation between both consequences of obesity.O

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